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Immunoassay Detection of Hepatitis B Surface Antigen Mutants

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The increasing use of hepatitis B vaccination has had an overwhelming positive impact on the prevention of hepatitis B viral infection. Mutations in the hepatitis B surface antigen (HBsAg) gene occur as a result of vaccine escape mutants, anti-hepatitis B surface antigen immunotherapy, or in chronic hepatitis B viral infection. These mutants may present a challenge to immunoassay detection. Evaluation of the immunodetection of various HBsAg mutants has been sporadic, as the occurrence of these mutants is not common, and sufficient volume of serum samples is difficult to obtain. To investigate mutant detection, recombinant antigens were constructed to reflect mutations described in the literature occurring throughout the S gene. A limited number of serum samples exhibiting discordant immunoassay reactivity were also used to construct recombinant antigens. The evaluation of 25 HBsAg mutants across nine commercial assays of differing formats is described. Mutations affecting immunoassay performance were characterized as occurring mainly in loop 2 of the "a" determinant of HBsAg. It was determined that reagent epitope recognition was more significant for mutant detection than assay format. ***J. Med. Virol. 59:19–24, 1999.***

KEY WORDS: vaccine escape mutants; HBsAg; variants; monoclonal antibody epitope

INTRODUCTION

Universal immunization against hepatitis B virus (HBV) is an effective and safe strategy for HBV control and prevention. The implementation of HBV vaccination programs is gaining worldwide acceptance by both health care professionals and the general population. The HBV vaccine is safe, immunogenic, and efficacious [Zuckerman, 1990]. However, administration of the HBV vaccine along with hepatitis B immunoglobulin has on rare occasions resulted in mutation of a group-specific determinant within the hydrophilic region (determinant "a") of hepatitis B surface antigen (HBsAg) [Zanetti et al., 1988; Carman et al., 1990]. These mutations are described as vaccine-escape mutants or variants that are formed by altered expression of HBsAg "a" determinant epitopes, which allows both infection in previously vaccinated individuals [Zuckerman et al., 1996] as well as a lack of detection by some commercially available HBsAg assays [Carman et al., 1995].

The production of antibody to HBsAg (anti-HBs) after either recovery from an acute HBV infection or immunization with HBV vaccine is directed against the "a" determinant of HBsAg, which is common to all subtypes of the virus. This determinant is located between amino acid (aa) residues 124 and 147 of HBsAg and is postulated to have a double loop structure [Brown et al., 1984]. The most prevalent HBsAg variant is the glycine to arginine mutation at aa position 145 of the second loop of the "a" determinant of HBsAg [Zanetti et al., 1988; Carman et al., 1990; Okamoto et al., 1992; Yamamoto et al., 1994; Zuckerman et al., 1994].

Other less prevalent mutations outside the double loop structure [Wallace et al., 1994; Carman et al., 1997] or within it [Oon et al., 1995] have been identified. These include some of the following mutations: cysteine to tyrosine at aa position 124; isoleucine/threonine to alanine or to serine at aa position 126; glutamine to histidine at aa position 129; methionine to leucine at aa 133; tyrosine to cysteine at aa position 137; threonine to serine at aa position 140; proline to serine or to leucine at aa position 142; and aspartic acid to alanine at aa position 144.

Also reported in the literature are other variants that occur to a lesser extent than the glycine to arginine mutation at aa position 145. These variants were presumably formed by other mechanisms such as deletions [Grethe et al., 1998] or insertions [Yamamoto et al., 1994; Hou et al., 1995; Carman et al., 1995] of amino acids in the "a" determinant. Besides vaccina-

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tion-associated HBsAg mutants, mutations in the HBV surface gene have been reported in patients with orthotopic liver transplantation on therapeutic trials with monoclonal anti-HBs [MacMahon et al., 1992], in a carrier who did not receive hepatitis B immunoglobulin or vaccine [Moriyama et al., 1991], and in chronic HBV carriers [Yamamoto et al., 1994] and other HBV carriers on antiviral therapy.

To compare the detection of HBsAg mutants in various immunoassays, we decided to produce recombinant HBsAg antigens containing defined point mutations throughout the "a" determinant. Recombinant HBsAg was also produced from DNA isolated from clinical samples suspected of harboring vaccine escape mutants. The advantage of this approach is that sufficient amounts of antigen can be generated and quantitated to a desired concentration. These antigens can then be evaluated across HBsAg assays to determine initial detectability followed by confirmation. The outcome of such testing would map the mutations affecting diagnostically important epitopes for HBsAg detection.

MATERIALS AND METHODS

Serum Samples

Serum samples were obtained from patients whose HBsAg diagnostic test results were discordant and indicated the potential presence of HBsAg mutants. Samples were extracted with phenol:chloroform:isoamyl alcohol after the protocol of Sambrook et al. [1989].

PCR Analysis

Nested primer sets were constructed to amplify the entire surface antigen gene from serum samples using the polymerase chain reaction (PCR) amplification. The first-round primers used were: 2813F-TCATT TTGTG GGTCA CCATA TT and 995R-TTGAC ATACT TTCCA ATCAA TAGG. The second-round primers were 2822F-GGGTCA CCATA TTCTT GGGAA C and 850R-GTTTT ATTAG GGTTT AAATG TAT. Perkin-Elmer GeneAmp kit reagents were used to amplify sequences using a Perkin-Elmer 9600 thermocycler. The PCR product was directly sequenced using a Perkin Elmer-ABI 373 automated sequencer. DNA sequences were analyzed using the Sequencer 3.0 software. For producing point mutations to reproduce samples described in the literature, the Stratagene QuikChange kit was used on an adw2 containing plasmid. Patient PreS2/S gene sequences were amplified with preS2 primer set 3135-Xho-F-GCGCG CCTCGA GCCAC CAATC GSCAG TCAGG AA and 850-Hpa-R GCGCG CGTTA ACGTT TTRTT AGGGT TTAAA TGTAT.

Cloning and Expression

Surface antigen gene sequences containing defined mutations were cloned into the *Xho*I and *Hpa*I restriction sites of a proprietary expression vector. Inserted genes were verified by sequencing, and the expression vector was used to transiently transfect mouse L cells. Cell culture supernatant was monitored for the expression of recombinant antigen. Expressed antigen was titrated to approximately 1 ng/ml in normal human serum previously screened to be HBsAg and anti-HBs negative. Titration experiments were performed with the Ausria II assay versus standardized HBsAg calibrators of known concentration.

Immunoassays

Panels of recombinant antigens along with negative and positive controls were coded and assayed using six Abbott HBsAg diagnostic kits, and with three commercially available HBsAg assays. The bead assays used were the Ausria II kit, which has a polyclonal capture solid phase and a polyclonal detection conjugate, and the Auszyme kit, which has a monoclonal capture solid phase and a monoclonal detection conjugate. The microparticle-based assays included IMx HBsAg, AxSYM HBsAg, and PRISM HBsAg, which have a monoclonal capture solid phase and a polyclonal detection reagent, and the ARCHITECT HBsAg which has a modified monoclonal capture solid phase and a polyclonal detection reagent.

Commercial assays A and B have both a monoclonal capture solid phase and a monoclonal detection conjugate. Commercial assay C has a polyclonal capture solid phase and a monoclonal detection conjugate. Confirmatory procedures were carried out on all reactive samples in their respective assays as per manufacturer's instructions.

RESULTS

Antigen Expression

The Ausria II assay was chosen specifically to quantitate the cell culture expression of HBsAg mutants because the polyclonal capture and detection reagents recognize a broad range of HBsAg antigens in the preS1, preS2, and S gene products. Therefore, destruction of a subset of epitopes by a vaccine escape mutation in the "a" determinant would have a minimal impact on quantitation. In addition, clinical experience had demonstrated the utility of Ausria II to detect HBsAg insertion mutants. Recombinant mutant proteins adjusted to 1ng/ml by Ausria II, reacted in other HBsAg assays with a signal equivalent to a 1 ng/ml standard if that antigen did not contain a mutation that affected the assay's reagents. This confirms the validity of the initial Ausria II quantitation.

Transient transfection of mammalian cells with plasmids containing the HBsAg sequence of interest in the expression vector, resulted in expressed antigen in the range of 25–100 ng/ml culture supernatant for the wild-type and most of the substitution mutants. An exception was the plasmid containing insertion mutants or multiple substitutions in the S gene product, which resulted in expressed antigen in the range of 1–5 ng/ml culture supernatant.

Immunoreactivity

Initially, 10 mutant antigens were expressed that reflected a known population and prevalence of HBsAg

TABLE 1. Detection of Recombinant HBsAg at 1 ng/ml From More Prevalent Mutants

HBsAg Mutants/Configuration	Ausria Poly/Poly	Auszyme Mono/Mono	IMx HBsAg Mono/Poly	AxSYM Mono/Poly	PRISM Mono/Poly	Architect Mono/Poly	Commercial Assay A Mono/Mono	Commercial Assay B Mono/Mono	Commercial Assay C Poly/Mono
wild type	++	++	++	++	++	++	++	++	++
Thr126- Ser	++	+	+	++	++	++	+	++	++
Gln129- His	++	+	+	++	++	++	+	++	++
Met133- Leu	++	++	++	++	++	++	++	++	++
Asp144- Ala	++	++	++	++	++	++	-	++	++
Gly145- Arg	++	++	++	++	++	++	-	-	-
Thr126- Ser + Gly145- Arg	++	++	+	++	++	++	-	-	-
Pro142- Leu + Gly145- Arg	++	++	++	++	++	++	-	-	-
Pro142- Ser + Gly145- Arg	++	++	++	++	++	++	-	-	-
Asp144- Ala + Gly145- Arg	++	++	++	++	++	+	-	-	-

Note: All positive samples confirmed in their respective assays.

Key: ++ = equivalent detection to wild type antigen
+ = detection less than wild type antigen

vaccine escape mutants described previously [Oon et al., 1995]. These mutants, occurring in the "a" determinant between aa 124–147 of the "S" gene product, were evaluated across nine immunoassays (Table I). The most common HBsAg vaccine escape mutant (glycine to arginine at aa position 145) was recognized universally and confirmed positive across the six Abbott assay configurations with a sensitivity equivalent to wild-type antigen. The commercially available assays A, B, and C were unable to recognize this mutant even at the highest concentration of antigen available for testing in the neat cell culture supernatant.

A second set of 20 mutant antigens were produced and evaluated across nine immunoassays (Table II). This second set of antigens included some substitution mutants described in the literature as occurring at a very low prevalence. Sufficient neat serum was available from one sample (proline to glutamine at aa position 120) to confirm that the recombinant HBsAg immunorecognition is equivalent to that of naturally occurring mutant, as has already been shown for diluted serum from a previously described insertion mutant [Carman et al., 1995]. The prevalence of these mutants cannot be stated with certainty, but they are presumed to be significantly less frequent than the glycine to arginine substitution at aa position 145.

A recent description of serum samples containing purported HBsAg variants with amino acid substitutions outside of the conventional "a" determinant that were negative by the Abbott HBsAg IMx assay [Carman et al., 1997] was investigated further by generating the corresponding point mutations. These three substitutions were evaluated in the IMx assay along with a control antigen (Table III). In this case, immunoreactivity of the IMx assay was equivalent to the wild-type antigen, suggesting that these purported variants do not present an altered epitope to the assay used to screen for them.

DISCUSSION

The data presented in Table I represent the sum of 16 vaccine escape mutant sequences identified in infants found in 345 births to HBsAg- and HBeAg-positive mothers [Oon et al., 1995]. Twelve of 16 of these infants had the glycine to arginine substitution at aa position 145 either individually or in combination with other mutations. Assay configuration itself was not the sole factor predicting mutant detection, as the Auszyme assay (monoclonal capture/monoclonal detection) readily detected the glycine to arginine substitution at aa position 145, while the Commercial assays A and B (both monoclonal capture/monoclonal detection) did not recognize this substitution. More importantly, it is the epitope(s) recognized by the reagents used to immobilize or detect antigen that determine whether HBsAg with the glycine to arginine substitution at aa position 145 or any other variant is discerned successfully. Reagent substitution experiments indicated that in the case of Commercial Assay A the immobilized solid phase antibody failed to capture the glycine to arginine substitution at aa position 145, while in Commercial Assay B and C the detection phase antibody failed to bind this mutant. While the glycine to arginine substitution at aa position 145 represents the major vaccine escape mutant, other mutants have been described in the literature as novel occurrences with unknown clinical prevalence. Table II presents the im-

TABLE 2. Detection of Recombinant HBsAg at 1 ng/ml From Less Prevalent Mutants

HBsAg Mutants/Configuration	Ausria Poly/Poly	Auszyme Mono/Mono	IMx HBsAg Mono/Poly	AxSYM Mono/Poly	PRISM Mono/Poly	Architect Mono/Poly	Commercial Assay A Mono/Mono	Commercial Assay B Mono/Mono	Commercial Assay C Poly/Mono
wild type	++	++	++	++	++	++	++	++	++
Asn40- Ser	++	++	++	++	++	++	++	++	++
Pro111- Thr	++	++	+	++	++	++	++	++	++
Thr Thr115,116- Ile Ile	++	++	++	++	++	++	++	+	++
Thr118- Ser	++	++	++	++	++	++	++	++	++
Pro120- Gln	++	-	++	++	++	++	-	++	++
Serum	++	-	++				-		
Thr131- Ile	++	++	++	++	++	++	++	++	++
Pro135- Ser	++	++	++	++	++	++	-	-	++
Lys141- Glu	++	-	++	++	++	++	-	-	++
Pro142- Leu	++	++	++	++	++	++	-	-	++
Pro142- Ser	++	++	++	++	++	++	++	-	++
Gly145- Ala	++	++	++	++	++	++	+	++	++
Gly145- Lys	++	++	++	++	++	++	-	-	-
Thr148- His	++	+	++	++	++	++	-	++	++
Ser154- Trp	++	+	+	++	++	++	-	-	-
MetMetMet196-198- SerSerSer	++	++	++	++	++	++	++	++	++
Clinical seqs.									
Insertion seq. A (adw2)	++	-	-	-	-	+	-	-	++
Insertion seq. B	++	-	-	-	-	-	-	-	-
Serum (ayw1)	++	-	-				-		

Note: All positive samples confirmed in their respective assays.

Key: ++ = equivalent detection to wild type antigen
+ = detection less than wild type antigen

TABLE 3. Detection of Recombinant HBsAg at 1 ng/ml From Purported Mutants

HBsAg Mutants	Ausria	IMx HBsAg
wild type	++	++
Phe134- Ala	++	++
Ser155- Tyr	++	++
Ala157- Arg	++	++

Key: ++ = equivalent detection to wild type antigen
+ = detection less than wild type antigen

munoreactivity of many of these point mutants. Substitutions in the proposed second loop of the "a" determinant between aa positions 138–147 appear to be less detectable by the monoclonal capture/monoclonal detection assay configurations. Curiously, both the proline to glutamine substitution at aa position 120 and the lysine to glutamic acid substitution at aa 141 [Karthigesu et al., 1994] affect the Auszyme and Commercial Assay A similarly through alteration in the antigenicity of the proposed second loop of the "a" determinant, as was demonstrated by monoclonal mapping studies (data not shown).

The proline residue at aa position 120 preserves a portion of the three-dimensional epitope presented on the second loop of the "a" determinant. Sufficient serum was available from a patient with the proline to glutamine substitution at aa position 120 to test several assays in parallel with the recombinant HBsAg containing the same mutation. As shown, the serum sample and the recombinant antigen gave similar reactivity patterns. Also shown in Table II are results from two clinical specimens containing insertion mutants. Insertion sequence A was isolated in a patient from Toledo, Ohio, and has an insertion of arginine and alanine at aa position 123 in an adw2 backbone. This insertion mutant was originally described in a Chinese patient with chronic hepatitis [Hou et al., 1995]. Insertion sequence B contains an asparagine and threonine insertion at aa position 123 plus a glycine to arginine substitution at aa position 145 in an ayw1 backbone that was originally described in an Indonesian patient [Carman et al., 1995]. Again, there was sufficient serum available from the latter patient to test as a dilu-

tion against several immunoassays. Ubiquitous destruction of epitopes is evident in these two insertion mutants as few HBsAg assays with a monoclonal-based configuration are capable of detecting them.

In contrast to the monoclonal-based assays, the Ausria radioimmunoassay (RIA) was capable of detecting all recombinant HBsAg mutant proteins, including the insertion mutants. Ausria has two immunoreactive components to HBsAg, a solid phase capture and a radiolabeled probe [Mushahwar and Brawner, 1992]. The capture phase is a polystyrene bead coated with guinea pig antisera recognizing preS1, preS2, and S gene products of HBsAg. The probe is ^{125}I labeled human anti-HBs, which also recognizes the preS1, preS2, and S gene products of HBsAg. Two recent reports [Jongerius et al., 1998; Tang et al., 1998] have claimed independently that certain S gene point mutations in the "a" determinant affect Ausria II recognition of surface antigen. The first report maintains that a glutamine to arginine substitution at aa 129 and a methionine to threonine substitution at aa 133 affects Ausria, while the second report maintains that a sequential threonine to threonine to isoleucine to asparagine substitution at aa 115–116 affects Ausria antigen detection. The preS gene sequence was normal in these samples. Table II presents data on the similar S gene point mutants glutamine to histidine substitution at aa 129, methionine to leucine substitution at aa 133, and the sequential threonine to threonine to isoleucine to isoleucine substitution at aa 115–116. In contrast to the findings of Jongerius et al. [1998] and Tang et al. [1998], these recombinant proteins containing mutations at the same aa site were readily detected by all current Abbott HBsAg assays (Ausria, Auszyme, IMx HBsAg, AxSYM HBsAg, PRISM HBsAg, and ARCHITECT HBsAg). In addition, recombinant preS2 antigens from insertion mutants A and B were also detected by Ausria even though many of the "a" determinant epitopes have been altered. Insertion mutants A and B were detected by Ausria due the preS1 and preS2 immunocomponents of both the kit capture and probe. The design of the Ausria assay is unlikely to be affected by vaccine escape mutants occurring in the "a" determinant. As shown in Table III, nonreactive serum samples in a particular assay do not necessarily harbor HBsAg mutants. The three HBsAg substitutions described were found in samples negative by IMx HBsAg but positive by an experimental monoclonal capture/polyclonal detection assay with unproven specificity [Carman et al., 1997]. Recombinant proteins containing these same substitutions were readily detected by IMx HBsAg with a sensitivity equivalent to the wild-type antigen. This suggests that an alternative mechanism is involved in the explanation of these results [Carman et al., 1997], such as sensitivity endpoint differences in the initial screening assays coupled with a possible generation of artifacts due to PCR amplification [Gunther et al., 1998].

In conclusion, the utilization of recombinant proteins to map enzyme immunoassay performance is useful for establishing defined, standardized samples to evaluate mutation susceptibility. This susceptibility testing must be interpreted in light of the prevalence of mutants being tested. It is important to confirm that lack of immuno-reactivity is due to a defined mutation in a sample by constructing the corresponding recombinant antigen to eliminate the potential effect of sample interference or degradation. Lastly, these antigens present a useful means of testing the robustness of HBsAg assay configurations.

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